Genetic Analysis of Mutations to Increased Virulence in *Magnaporthe grisea*

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ABSTRACT

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An isolate of *Magnaporthe grisea*, 70-14, virulent on 10 cultivars of rice, but avirulent on rice cultivar Katy, was mutagenized with nitrosoguanidine. Four mutants of independent origin that showed an increase in virulence on Katy were recovered. The virulence on the other 10 rice cultivars was not affected. The lesion size caused by these mutants was intermediate between the parent avirulent isolate, 70-14 (infection type 1), and a virulent isolate, 70-6 (infection type 4). The numbers of lesions also decreased at least 10-fold when compared to numbers of lesions caused by a virulent wild-type isolate. A range of lesion types (0–4) was common in these mutant isolates, with both small and large lesions. In contrast, wild-type virulent isolates always gave large lesions. The lesions of the mutants had a wide, brownish lesion edge compared to the water-soaked appearance of lesions of a virulent wild-type isolate. Genetic analysis showed that in two of the mutants, one of the two avirulence genes in 70-14, *P12*, was mutated. A new locus, *M*, was mutated in the other two isolates and identified as being required for the expression of avirulence genes. In addition, a mutation of the suppressor, *S11*, of the fourth mutant also may have been recovered.

Additional keywords: Avirulence/virulence genes, host–parasite interactions.

The gene-for-gene hypothesis successfully accounts for the pattern of interaction between resistance genes (*R*) in rice (*Oryza sativa* L.) and genes conferring specific avirulence/virulence (*P* genes) in *Magnaporthe grisea* (T.T. Hebert) Barr ((*Pyricularia grisea*) Sacc. (=*Pyricularia oryzae* Cavara)) (3–5,7,9,10). The interaction between a host *R* gene and a corresponding pathogen *P* gene results in incompatibility (low-infection type), and the interaction is genetically specific.

Induced mutations to increased virulence have been reported in many pathogens (2). Mutations to reduced virulence on specific-host cultivars are rare but have been obtained (8). One explanation for the rare recovery of mutations showing cultivar-specific reduced virulence is that it is difficult to screen for mutations to decreased virulence. Because mutations usually have deleterious effects on genes, the frequency of forward mutations (losses of function) is almost always higher than that of reverse mutations (regaining of function). Because avirulence is the active and dominant function, mutations to increased virulence against specific-host resistance genes (*R*) should be more frequent than mutations to decreased virulence on cultivars with specific *R* genes.

Isolate 70-14 of *M. grisea* is a highly fertile hermaphrodite (1). It also is highly pathogenic and virulent on 10 rice cultivars. Segregation of avirulence/virulence on rice cultivar Katy was observed among progenies of crosses with isolates virulent on Katy. Our studies have shown that isolate 70-14 contains two avirulence genes, *P11* and *P12*, and the suppressor, *S11*, of the *P11* gene (5). The objective of the research described here was to obtain mutations to increased virulence, some of which would be at the *P12* locus and some of which would be at other loci that affect the expression of avirulence. One goal was to obtain many mutations at the *P12* locus to develop an allelic series at that locus. This paper reports on the analyses of four mutants of isolate 70-14 that have increased virulence on rice cultivar Katy.

MATERIALS AND METHODS

Fungal strains. Isolates 70-6 and 70-14 of *M. grisea* were described by Chao and Ellingboe (1). Guy11 was described by Leung et al (7).

Culture maintenance and media. Oatmeal agar (1,3–5) was used for the matings and for production of conidia. Complete and minimal media were as described previously (9,10). Complete media plus 3% sorbose was used for the determination of percent viable conidia after mutagenesis and for replica plating. For long-term culture storage, cultures were grown on complete media for 5–6 days at 22–25 C, air-dried in a sterile hood, and stored at −20 C (1,3). For short-term storage, (2–4 wk), isolates were grown on complete medium agar slants and stored at 4 C.

Crossing. Crosses between mutants and 70-6 or Guy11 were made by placing mycelia in agar blocks with the two isolates about 5 cm apart on oatmeal agar in a petri plate and incubating at 22–24 C (6). Mature perithecia usually formed after 14–21 days. Individual perithecia were removed, crushed, and dragged across the agar surface of complete media, a procedure that left a trail of asci on the agar surface (3,4). Individual asci were separated with a bent glass needle. The asci with germinated ascospores were picked and transferred to oatmeal agar. The colonies produced conidia in about 3–4 days. One conidium was isolated from each ascus colony, ensuring the collection of one progeny from each meiotic event (1,3).

Nitrosoguanidine mutagenesis. Isolate 70-14 was grown for about 10 days on oatmeal agar plates. The plates were flooded with 10 ml of sterile water, and conidia were scrapped off the agar surface with a bent sterile dissecting needle. The conidial suspension was filtered through two layers of Miracloth into a 250-ml Erlenmeyer flask. The final spore concentration was adjusted to 10^5 with a hemacytometer. The amount of colony forming units in the culture was determined by dilution plating onto 3% sorbose complete medium. This served as a control for the kill rate of the mutagenized culture. A filtered, sterilized solution of *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was added to a final concentration of 100 μg/ml. The flasks were wrapped with aluminum foil and shaken at 100 cycles/min for 30 min. The mutagenized culture was dilution plated onto 3% sorbose complete medium to determine the killing rate. Auxotrophic mu-

tants were screened by replica plating the master plate onto 3% sorbose minimal medium with sterile Whatman filter paper. For temperature-sensitive mutants, the master plate was replica plated onto a 3% sorbose complete medium and incubated for 24 h at 16 or 32 C. The filter paper was then removed, and incubation was continued for another 24 h. The frequency of auxotrophic and temperature-sensitive mutants was used as an indicator of the efficacy of the mutagenesis. The auxotrophic and temperature-sensitive mutants were not tested for pathogenicity. Two separate mutageneses were performed.

Plant materials. Rice seeds were obtained from M. A. Marchetti and A. M. Clung, USDA-ARS, Beaumont, TX; S. Linscomb, Rice Research Station, Crowley, LA; K. Moldenhauer, Rice Research and Extension Center, Stuttgart, AR; and D. M. Brandon, Rice Experimental Station, Biggs, CA. The cultivars used in this study were S201 (CI 9974), Bluebelle (CI 9544), L202 (PI 483097), Leah (CI 9979), Katy (PI 527707), Lebonnet (CI 9882), Lemont (PI 475833), M201 (CI 9980), M103 (PI 527566), Newbonnet (PI 474580), and Maybelle (PI 538248).

Test of pathogenicity. Approximately 1,000 colonies, each derived from a single mutagenized conidium, were picked from sorbose complete medium and tested for altered virulence. In the initial test, 10–20 colonies were grown together as a pool on single oatmeal plates. The conidia harvested from these plates were adjusted to 10^5 spores per milliliter and inoculated onto 15–20 seedlings of each of 11 rice cultivars at the two- to three-leaf stage. The inoculated plants were covered with plastic bags and incubated in a moist chamber for 24–36 h before transfer to a greenhouse. The infection type (IT) was determined seven days after inoculation. The scale of reactions was as described previously (3–5). Four ITs were recognized: 1 = minute black spots, 2 = black lesions 2–3 mm in length, 3 = usually circular lesions with gray centers, and 4 = large, diamond-shaped water-soaked lesions, commonly coalescing into long stripes with gray centers. The ITs produced by 70-14 (IT 0–1) and 70-6 (IT 3–4) on Katy were clearly distinct, and the classification of progenies into virulent and avirulent classes was unequivocal (5). If there were any IT 3 or 4 lesions recovered from the inoculations with mutagenized isolates, the individual isolates from the pool were individually recultured from the stock cultures and inoculated separately to determine which individual in the pool showed an increase in virulence. Finally, a single conidium was isolated from one IT 3 or 4 lesion and retested for virulence. Inoculations were done in a protocol that minimized the chances of cross-contamination between isolates. Inoculations with sterile water gave no lesions. Once the virulence of the individual mutants was confirmed by repeating the pathogenicity tests, the mutants were crossed with 70-6 and Guy11 for genetic analysis.

Crosses. Five isolates, L74, L129, L629, L631, and L637, were crossed with 70-6 and Guy11, both of which are highly virulent on Katy. Isolate 70-6 is a sibling of 70-14, whereas Guy11 is one of the three original parents in our breeding program that led to the development of 70-6 and 70-14 (1). Isolate 70-14 carries two avirulence genes, *P11* and *P12*, and a suppressor, *S11*, of *P11*, whereas 70-6 and Guy11 carry *p11 p12 s11 S12* and *p11 p12 S11 s12*, respectively (5). (A lowercase *p* or *s* indicates a recessive allele.) Isolate 70-14 is avirulent due to the presence of *P12*. Though 70-14 contains *P11*, it also contains *S11*, which suppresses the expression of avirulence due to *P11*. Mutations to increased virulence are expected to be at the *P12* locus or at a locus that is necessary for the expression of avirulence controlled by *P12*.

RESULTS

Culture treatment for 30 min with 100 μg of NTG killed 95% of the conidia. Of about 4,000 individual conidial colonies tested, 19 were auxotrophic, and 35 were high-temperature sensitive. Ten buff mutants were recovered. These mutants showed that the mutagenesis procedure was successful. No auxotrophic or temperature-sensitive mutants were obtained in the controls.

Fifteen individual isolates were obtained from large lesions on Katy. All 15 isolates were basically unchanged in their virulence on 10 rice cultivars, except possibly on cultivar M201 (Table 1). The virulence of these mutants changed from the avirulence of isolate 70-14 to an intermediate virulence on Katy (Table 1). The term intermediate is used to indicate that a range of infection types was observed (Table 1). No mutants were to full virulence with ITs 3–4, as observed in wild-type virulent isolate 70-6. The phenotype of the mutants was clearly distinguishable from the phenotype of the parent isolates 70-14 and 70-6 on Katy. The isolates were easily and repeatedly classified into the three classes: avirulent (like 70-14), virulent (like 70-6), and intermediate. The intermediate class varied more between replicates but was easily distinguishable from the avirulent and virulent siblings.

The lesions produced on Katy after inoculation with 70-6 (wild-type and virulent on Katy), 70-14 (wild-type and avirulent on Katy), and L629 (intermediate), scored after 7 days of inoculation, are given in Figure 1. Several differences were clear. Parent isolate 70-14 produced no large lesions on Katy. The largest lesions produced by L629 (Fig. 1B and C) were approximately 30% smaller than the lesions produced by isolate 70-6 (Fig. 1D and E). Lesions produced by the mutants also were commonly associated with wide, brownish borders (Fig. 1B). Brown borders were either much thinner or absent from the lesions produced by 70-6 (Fig. 1D). The gray center in the mutant (Fig. 1B) also

TABLE 1. Infection types of isolate 70-14 of *Magnaporthe grisea* and 15 mutants derived from 70-14 that increased virulence on rice cultivar Katy

Isolate or mutant designation	Rice cultivar										
	Bluebelle	L202	Leah	Lebonnet	Lemont	M103	M201	Maybelle	Newbonnet	S201	Katy
70-14	4[a]	4	4	4	4	4	2–4+[b]	4	4	4	1
L74	4	4	4	4	4	4	4	4	4	4	3
L105	3	4	3–4+	4	4	4	2–4+	4	4	4	1+–4
L108	4	4	4	4	4	4	2–4+	4	4	4	1+–3
L129	3	4	4	4	4	4	4	4	4	4	1+–3
L207	4	4	4	4	4	4	4	4	4	4	1+–4
L253	4	3	4	3	4	4	2–4+	4	4	4	2+–4
L271	4	4	4	4	4	4	2–4+	4	4	4	2+–4
L274	3	3–4	4	4	4	4	4	4	4	4	2+–4
L289	4	4	4	4	4	4	2–4+	4	3	3	2+–4
L290	4	4	4	4	4	4	4	4	4	4	1+–3
L470	3	3	4	4	4	4	4	3+–4	4	4	1+–4
L629	4	4	4	4	4	4	2–4+	4	4	4	1+–3
L631	4	4	4	3	4	4	4	4	4	4	1+–4
L637	4	4	4	4	4	4	4	4	4	4	1+–3
L791	4	4	4	4	4	4	4	4	4	4	1+–3

[a] Infection types 2–4+, 3–4, 3, and 4 are considered virulent.
[b] + indicates the predominant infection type.

was much smaller than in virulent isolate 70-6 (Fig. 1E). The water-soaked phenomenon exhibited by the lesion (Fig. 1E) was not visible in the mutants.

The mutants always incited 10–50 times fewer large lesions than small lesions on the same plants (Fig. 1C). The number of large lesions ranged from three to a maximum of about 10 on any one leaf. No coalescing of lesions was ever observed for the mutants as was observed in the virulent isolate 70-6 (comparison of Fig. 1B and C with E). Several isolates were obtained from single conidia from large lesions. These isolates were inoculated onto Katy. The same range of lesion types was observed for each isolate. This observation, coupled with differences in lesion morphology of the mutants, showed that the increase in virulence of the mutants was not due to the contamination of the parent isolate. The ability of mutants to infect the host seemed unaffected because they were still capable of causing as many lesions as was avirulent wild-type 70-14 (comparison of Fig. 1C and A).

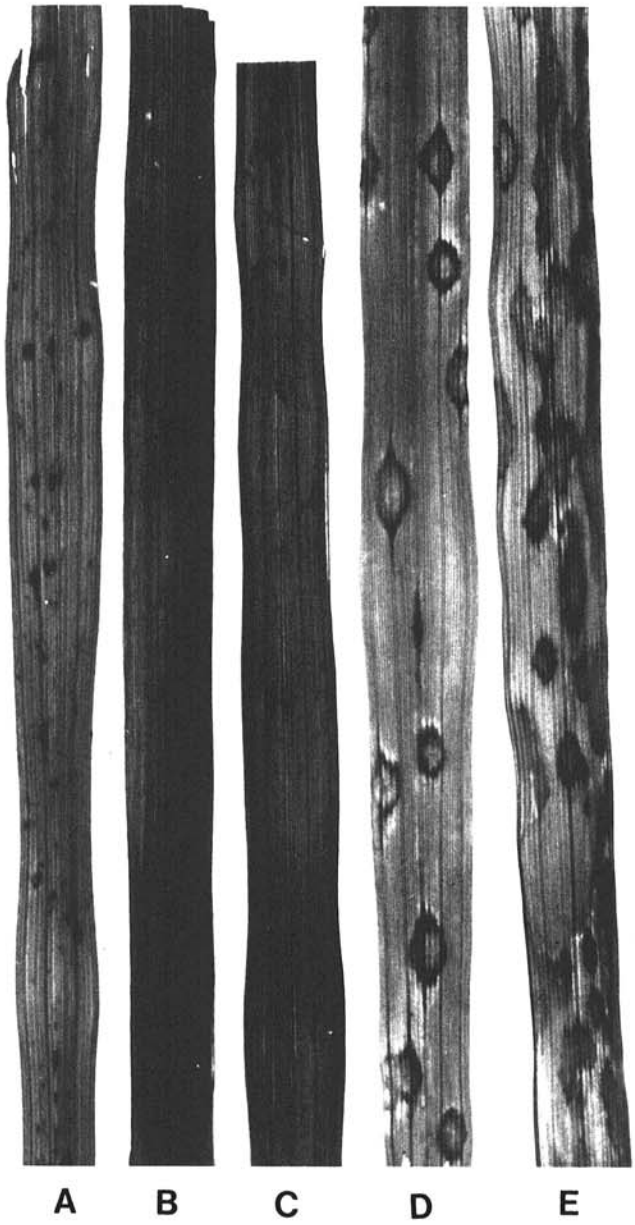

Fig. 1. Infection type of isolate **A,** 70-14, **B and C,** mutant L629, and **D and E,** 70-6 on cultivar Katy 7 days after inoculation.

Mutant isolates L129, L629, L631, and L637 crossed readily with both 70-6 and Guy11. Isolate L74, however, failed to cross with Guy11 and was dropped from further analyses. If increases in virulence were caused by a leaky mutation, *P12**, at the *P12* locus, a cross between the mutant and 70-6 (*P11 P12* S11 s12* × *p11 p12 s11 S12*) would allow segregation at all four loci, and a ratio of 4 avirulent:3 intermediate:9 virulent would be expected to be recovered after inoculation on Katy. A cross between a leaky mutation at *P12* with Guy11 (*P11 P12* S11 s12* × *p11 p12 S11 s12*) should show no segregation of avirulence/virulence due to *P11* because the cross is homozygous for *S11* but is expected to segregate 1 intermediate (*P12**):1 virulent (*p12*) on Katy.

The progenies from crossing mutant L629 with 70-6 segregated 14 avirulent:16 intermediate:29 virulent (Table 2). When this same mutant was crossed with Guy11, the progenies segregated 19 intermediate:12 virulent. Similar results were obtained with L637 (Table 2). The results suggest that the mutations from avirulent to intermediate virulence in these two mutants occurred at the *P12* locus.

The segregation from crosses of L129 and L631 with 70-6 and Guy11 was different from the segregation in crosses with L129 and L637 (Table 2). Avirulent progenies were obtained when L129 was crossed with either 70-6 or Guy11. The recovery of avirulent progenies in the cross with Guy11 suggests that the mutation in L129 that led to increased virulence was not at the *P12* locus. If the mutation occurred at a second locus, *M*→*M**, the cross of L129 with Guy11 (*P11 P12 S11 s12 M** × *p11 p12 S11 s12 M*) would give four types of progenies, namely, *P12 M* (avirulent), *P12 M** (intermediate), and *p12 M* and *p12 M** (virulent). The observed segregation ratio of 2 avirulent:4 intermediate:6 virulent (Table 2) is a good fit to a calculated ratio of 1:1:2.

The isolate 70-14 has been shown to differ from 70-6 by four genes: two avirulence genes and two suppressors of the avirulence genes (5). Their genotypes are: 70-14, *P11 P12 S11 s12*, and 70-6, *p11 p12 s11 S12*. The segregation ratio of the progenies from the cross 70-14 × 70-6 is 7 avirulent:9 virulent. The segregation ratio among progenies of L129 × 70-6 is 6 avirulent:6 intermediate:18 virulent. The proportion of progenies with some degree of avirulent/virulent progenies (12:18) is a reasonable fit to a 7:9 ratio. The ratio of avirulent progenies/intermediate progenies is 1:1. Therefore, the data suggest that the mutation *M** to intermediate avirulence is affecting both *P11* and *P12*. If the mutation *M** affects only *P12*, the expected segregation ratio

TABLE 2. The segregation of avirulence/virulence on Katy in crosses of mutant derivatives of isolate 70-14 with isolates 70-6 and Guy11

Cross	Parent isolate 1	Parent isolate 2	IT of parent[a] 1	IT of parent[a] 2	Number of progeny with each IT 0–1	1–2	1^{+b}–3	1^{+}–4	2^{+}–3	2^{+}–4	3–4	Observed ratio (avir:int:vir)[c]	Predicted ratio
263	70-6	L129	4	1^{+}–3	1	5	0	6	0	0	18	6:6:18	$x^2_{7:7:18} = 0.17$
264	Guy11	L129	4	1^{+}–3	0	2	3	1	0	0	6	2:4:6	$x^2_{2:2:4} = 0.66$
265	70-6	L629	4	1^{+}–3	1	13	4	9	3	0	29	14:16:29	$x^2_{4:3:9} = 2.77$
266	Guy11	L629	4	1^{+}–3	0	0	8	11	0	0	12	0:19:12	$x^2_{0:1:1} = 1.58$
267	70-6	L631	4	1^{+}–4	7	2	0	8	0	0	9	9:8:9	$x^2_{5:5:6} = 0.92$
268	Guy11	L631	4	1^{+}–4	4	1	8	3	1	0	12	5:12:12	$x^2_{5:5:6} = 2.89$
269	70-6	L637	4	1^{+}–3	11	1	6	6	0	0	22	12:12:22	$x^2_{4:3:9} = 1.92$
270	Guy11	L637	4	1^{+}–3	0	0	8	8	0	0	16	0:16:16	$x^2_{0:1:1} = 0.00$

[a] Infection types (ITs) 1–2 are considered avirulent. ITs 1–3, 1–4, 2–3, and 2–4 are considered intermediate. ITs 3–4 are considered virulent.
[b] + indicates the predominant IT.
[c] Avirulent:intermediate:virulent.

would be 14:7:27, which is also a reasonable fit to the observed 6:6:18 ratio ($X^2 = 1.54$).

If the mutation to increased virulence in L631 is at the *P12* locus, then the segregation of the progenies of the cross of L631 × Guy11 should be 1 intermediate:1 virulent. Avirulent progenies were recovered from this cross. The observed segregation ratio was 5 avirulent:12 intermediate:12 virulent progenies. The mutation that leads to increased virulence must be at a locus other than *P12*. When mutant L631 was crossed with 70-6, (*P11 P12 S11 s12 M* × p11 p12 s11 S12 M*), the progenies segregated 9 avirulent:8 intermediate:9 virulent. The observed segregation was 17 with some degree of avirulence (9 avirulent + 8 intermediate):9 virulent. The segregation of 17:9 was clearly a significant deviation from 7 avirulent:9 virulent obtained in the cross 70-14 × 70-6 (5). An explanation for the data must account for the change to intermediate virulence of 70-14 and the increase in the proportion of avirulent progenies from the cross L631 × 70-6. The cross of L631 with Guy11 showed that the mutation that gave increased virulence on Katy was at a locus other than *P12*. With two suppressors segregating in the cross L631 × 70-6, 9/16 of the progenies are expected to be virulent. To obtain an increase in the proportion of avirulent progenies, it is necessary to postulate the presence of a second mutation in L631. Two possibilities include inactivation of the *S11* suppressor in 70-14 or the activation of a previously silent avirulence gene in 70-14. For example, if the mutagenesis of 70-14 leads to a mutation at both the *M* and the *S11* loci (*P11 P12 S11 s12 M → P11 P12 S11* s12 M**), then the expected ratio of avirulent/intermediate/virulent in crosses of L631 with either Guy11 or 70-6 would be 5 avirulent:5 intermediate:6 virulent. The observed data agree with this ratio.

DISCUSSION

In the gene-for-gene hypothesis (2), resistance exhibited by an *R* gene is determined by the presence of a specific corresponding *P* gene of the pathogen. Resistance and avirulence are considered active functions. Hence, a mutation from avirulence to virulence would be from cultivar specificity to a loss of that specificity and should be easily obtainable.

Four mutants to increased virulence on rice cultivar Katy have been recovered after mutagenesis with NTG. Two of the mutations (in L629 and L637) appeared to be at the *P12* locus. Both gave intermediate phenotypes on Katy. Crosses with two virulent isolates, Guy11 and 70-6, gave progenies that showed segregations of intermediate and virulent reactions on Katy. Two mutants, L129 and L631, gave some avirulent progenies when crossed with either Guy11 or 70-6. The recovery of avirulent progenies indicates that the mutation to increased virulence was not at the *P12* locus, but at another locus we have tentatively designated *M*. Because the mutation at the *M* locus gave increased virulence, we concluded that the *M* locus has a function for the expression of avirulence. Mutations at either *P12* or *M* gave increases in virulence. Whether a mutation to increased virulence mapped to the *P12* or the independent *M* locus could be determined only by crossing. The *M* locus mimics the *P12* locus but, from our data, does not appear to have a phenotype in the presence of *p12*. The *M** mutation may also be important in the expression of *P11*, but the data are not conclusive as to whether *M** affects the expression of more than one *P* gene.

The crosses involving mutant L631 suggest that mutations occurred at two loci. One mutation to increased virulence was at a locus other than *P12* to give increased virulence on Katy. We postulated a second mutation to account for the increase in the proportion of progenies, in crosses with both Guy11 and 70-6, that are avirulent on Katy. If it is assumed that L631 contains a mutation of a locus (*M'*) that is necessary for the expression of both *P11* and *P12* and a second mutation that inactivates the *S11* locus, then it is possible to explain the origin of avirulent progenies in crosses of L631 with both Guy11 and 70-6, as well as the segregation ratios observed in these crosses.

The results presented here provide evidence for the segregation of three classes of genes: avirulence genes (*P* genes), suppressors of avirulence genes (*S* genes), and genes whose functions are necessary for the expression (not suppression) of avirulence (*M* genes). The mutations in L629 and L637 gave a change in reaction only on cultivar Katy (Table 2). The segregations in crosses of these mutants with 70-6 are consistent with the segregation of the locus designated *M** plus two avirulence genes and two suppressors (5).

The recovery of mutants of a locus or loci that are necessary for the expression of cultivar-specific avirulence is considered especially important. Not only do the data suggest the beginning of a pathway leading to cultivar-specific avirulence, but the *M* locus (or loci) could be misinterpreted as an avirulence gene. Mutations of the *M* locus lead to an increase in virulence, similar to the mutations of an avirulence gene.

The interdependence of the three classes of genes that control host cultivar-specific interactions is clear. The segregation of the *M* locus cannot be detected in the presence of the *p12* allele. The segregation at the *S12* locus cannot be detected in the presence of the *p12* allele. The segregation at the *P12* locus cannot be detected in the presence of the *S12* allele but can be detected in the presence of the mutant *M* locus. Mutations to loss or reduced function should give increases in virulence if at *P* loci but should give decreases in virulence if at *S* loci. An understanding of the interdependence of the three classes of genes is crucial to understanding the phenotype of a pathogen with a host plant.

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